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The activation of eukaryotic initiation factor (eIF)2B by growth factors in PC12 cells requires MEK/ERK signalling

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Abstract Epidermal and nerve growth factors (EGF and NGF) activate protein synthesis and initiation factor eIF2B in rat phaeochromocytoma (PC12) cells. The activation of protein synthesis by EGF or NGF depends upon extracellular regulated kinase kinase (MEK)/extracellular regulated kinase signalling. Here we show that PD98059, an inhibitor of MEK activation, blocks the activation of eIF2B by EGF or NGF. It is known that eIF2B activity can be inhibited by phosphorylation at Ser535 in its ε-subunit by glycogen synthase kinase (GSK)-3. We find that inactivation of GSK-3 by EGF or NGF is blocked by PD98059. However, neither EGF nor NGF caused a detectable change in phosphorylation of Ser535 of eIF2Bε. Thus, the EGF- and NGF-induced activation of eIF2B in PC12 cells involves regulatory mechanisms distinct from dephosphorylation of the GSK-3 site.

Key words: Eukaryotic initiation factor (eIF)2B; Glycogen synthase kinase-3; Extracellular regulated kinase

1. Introduction

Eukaryotic initiation factor (eIF)2B plays an important role in the initiation of mRNA translation in eukaryotic cells and in its control. eIF2B is a guanine nucleotide exchange factor and catalyses the exchange of GDP for GTP on eIF2. When complexed with GTP, eIF2 is in the active form and recruits the initiator Met-tRNA, which is required for recognition of the start codon, to the 40S subunit. The GTP is subsequently hydrolysed, leading to release of inactive eIF2·GDP complexes from the ribosome. In order to regenerate active eIF2·GTP, which is required for all translation initiation events, the GDP must be exchanged for GTP and this step is mediated by eIF2B.

The activity of eIF2B may be regulated in several ways. The best characterised mechanism involves phosphorylation of the α-subunit of eIF2, which leads to eIF2(αP) becoming a potent competitive inhibitor of eIF2B, and to inhibition of translation initiation [1]. Phosphorylation of the ε-subunit of eIF2B provides another mechanism for the regulation of the activity of eIF2B. Phosphorylation by glycogen synthase kinase-3 (GSK-3) inhibits the activity of eIF2B [2,3], while phosphorylation by casein kinase I or II is reported to increase eIF2B activity [4,5]. Inactivation of GSK-3 coincides with the activation of eIF2B in insulin-stimulated CHO-T cells and epidermal growth factor (EGF)- or nerve growth factor (NGF)-treated rat phaeochromocytoma (PC12) cells [6,7]. Furthermore, GSK-3 is regulated through phosphatidylinositol 3-kinase (PI 3-kinase) and a requirement for PI 3-kinase for the activation of eIF2B has been shown in several studies [6,7]. In addition, Ser540, the phosphorylation site for GSK-3 in eIF2Bε, undergoes dephosphorylation in vivo after insulin stimulation of CHO-T cells, and this is blocked by inhibitors of PI 3-kinase [3].

In our previous study [7], we described the regulation of protein synthesis, the activation of eIF2B and the inactivation of GSK-3 in response to EGF and NGF in PC12 phaeochromocytoma cells. In these cells, activation of eIF2B and inactivation of GSK-3 correlated with increased protein synthesis, which were all inhibited by wortmannin. Recently, we discovered that beside the PI 3-kinase dependent pathway, the activation of protein synthesis by EGF or NGF also involves extracellular regulated kinase kinase (MEK)/extracellular regulated kinase (ERK) signalling [8]. Given the previously demonstrated correlation between protein synthesis and eIF2B activation, we wished to examine the role of ERK signalling in the regulation of the activation of eIF2B. Our studies reveal, for the first time, that the MEK/ERK pathway is required for activation of eIF2B in PC12 cells. Furthermore, MEK-dependent activation of eIF2B does not involve changes in the phosphorylation state of the 'priming site', Ser539, or dephosphorylation of Ser535, the phosphorylation site for GSK-3 in rat eIF2Bε.

2. Materials and methods

2.1. Cell culture

PC12 cells were maintained as described previously [8]. The cells were grown to 60–70% confluency, starved of serum for 2 h, followed by treatment with EGF (50 ng/ml) or NGF (30 ng/ml) in the absence or presence of PD98059 (50 μM) and harvested in the buffer appropriate for the assay.

2.2. Measurement of protein kinase B (PKB) kinase activity

Cells were treated with EGF or NGF and harvested as described [8]. Antibodies directed against the three PKB isoforms, α, β and γ, were bound simultaneously to protein G, and about 100 μg of cell extract was used for each immunoprecipitation reaction. The immunoprecipitation and the PKB assays were performed as described previously [9].

2.3. Measurement of eIF2B activity

The eIF2B assay was performed as described previously [7]. Briefly, the cells were harvested in a buffer containing 20 mM Tris–HCl pH

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Abbreviations: EGF, epidermal growth factor; eIF, eukaryotic initiation factor; ERK, extracellular regulated kinase; GSK-3, glycogen synthase kinase-3; NGF, nerve growth factor; MEK, ERK kinase; PI 3-kinase, phosphatidylinositol 3-kinase; PKB, protein kinase B

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7.5, 50 mM β-glycerophosphate, 100 mM KCl, 0.2 mM sodium orthovanadate, 0.2 mM EDTA, 0.2 mM EGTA, 1% Triton X-100, 10% glycerol, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml antipain, 1 mM benzamidine and 1 mM DTT. Three μg of cell extract was used in each assay.

2.4. Measurement of GSK-3 activity

Antibodies against both the α- (Upstate Biotechnology Incorporated) and the β-isoforms (Transduction Laboratories) of GSK-3 were bound to protein G and used to immunoprecipitate GSK-3 from about 100 μg of cell extract. GSK-3 activity was determined as described [7], except that immunoprecipitated material, rather than crude cell extracts, was used.

2.5. Analysis of the phosphorylation state of ERK

After treatment, cells were harvested in Laemmli sample buffer and samples were analysed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) as described earlier [10]. ERK2 protein was detected by Western blotting with a polyclonal antibody raised in sheep (a kind gift from Dr. Dario Alessi, Dundee, UK).

2.6. Phosphorylation of eIF2Bε

Cell extracts (30 μg) were analysed by SDS–PAGE and Western blotting. Phosphorylation of Ser535 (which corresponds to Ser540 in the rabbit sequence) in rat eIF2Bε was monitored by using a phospho-specific antibody [3]. Phosphorylation of Ser539 (which corresponds to Ser544 in the rabbit sequence), the 'priming site', was detected using a phospho-specific antibody. The specific recognition of the phosphorylated Ser539 was examined using eIF2Bε that was either phosphorylated at this site or not (Maarten Janmaat and Chris Proud, unpublished observations). Equal loading was confirmed using an antibody that detects eIF2B irrespective of its state of phosphorylation.

3. Results

3.1. Activation of eIF2B by NGF or EGF requires the ERK pathway

PC12 cells were treated with EGF or NGF in the absence or presence of the MEK inhibitor PD98059 (50 mM) and the activity of eIF2B was measured. As described earlier [7], treatment of PC12 cells with NGF or EGF led to activation of the nucleotide-exchange activity of eIF2B (Fig. 1A). A specific inhibitor of MEK, PD98059, completely inhibited the ability of EGF or NGF to activate eIF2B, without affecting the basal level of eIF2B activity in control cells. These data imply that MEK, and thus ERK, signalling is required for the activation of eIF2B by EGF and NGF in PC12 cells.

3.2. Two different pathways are involved in the regulation of eIF2B

In order to verify that PD98059 does indeed block ERK signalling in PC12 cells, we assessed the phosphorylation state of ERK. Both EGF- and NGF-induced phosphorylation of ERK2 in PC12 cells, as manifested by a shift in the electrophoretic mobility of ERK2 (Fig. 1B) [10,11]. As expected, PD98059 completely blocked the phosphorylation of ERK2 in cells treated with either EGF or NGF.

It has been shown that in some cell lines wortmannin blocks the activation of ERK [12,13]. Therefore, it was important to establish that activation of eIF2B by EGF or NGF via a MEK-dependent pathway is different from the wortmannin-sensitive pathway previously described [7]. In PC12 cells, it was previously shown that wortmannin only partially blocks ERK phosphorylation [10]. We confirm this finding (Fig. 1B) when we treated the cells with EGF or NGF in the absence or presence of wortmannin (20–40% inhibition), showing that the effect of wortmannin on the activation of eIF2B is mainly via the PI 3-kinase pathway.

The PI 3-kinase dependent pathway involved in the regulation of eIF2B activity is thought to include GSK-3. Because PKB is a well-established regulator of GSK-3 [14,15], it was also important to exclude the possibility that PD98059 affected the activation of PKB by EGF or NGF to confirm the specificity of this inhibitor. PKB was rapidly activated after EGF or NGF treatment as reported earlier [8,16] and this activation was not affected by the MEK inhibitor PD98059 (Fig. 2). Thus, PD98059 only affects the MEK/ERK pathway, while the effect of wortmannin on the activation of eIF2B is mainly due to the PI 3-kinase pathway, indicating that two separate pathways are involved in the regulation of eIF2B.

3.3. The inactivation of GSK-3 is blocked by PD98059

In PC12 cells, both EGF and NGF induce the inhibition of GSK-3 concomitantly with the activation of eIF2B [7]. In view of our finding that the activation of eIF2B by these stimuli is blocked by PD98059, we studied whether PD98059 affected the ability of EGF and NGF to inactivate GSK-3 (Fig. 3A). As reported earlier [7], inactivation of GSK-3 by EGF or NGF is modest (reduction in activity of about 20–30% versus the control). In the presence of PD98059, the basal activity of GSK-3 was slightly reduced in control cells. However, in the presence of PD98059, neither EGF nor NGF

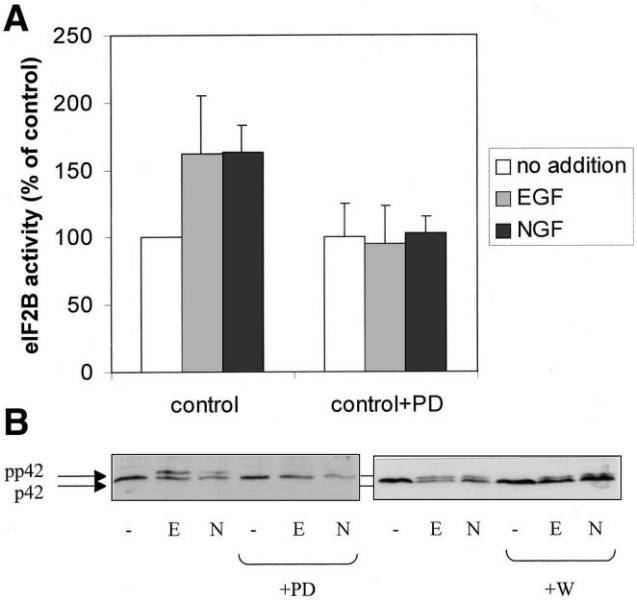

Fig. 1. Activation of eIF2B by EGF or NGF is regulated in an ERK-dependent manner. A: Cells were starved for serum and treated with EGF (60 min) or NGF (90 min) in the absence (control) or presence (control+PD) of PD98059. The cells were harvested and an eIF2B assay was performed as described in Section 2. Activation of eIF2B by EGF or NGF in the absence of PD98059 is significantly different from activation of eIF2B in the presence of PD98059 ($P \leq 0.05$). The eIF2B activity in control cells with no growth factors added was set at 100%. The error bars represent the standard error of the mean ($n = 5$). B: The cells were treated with EGF for 5 min or with NGF for 10 min in the absence or presence of PD98059 or in the absence or presence of wortmannin (100 nM). The cells were harvested in Laemmli sample buffer and the lysates were analysed by SDS–PAGE and Western blotting ($n = 3$). −, no addition; E, EGF added; N, NGF added. p42 and pp42 indicate, respectively, unphosphorylated and phosphorylated ERK2.

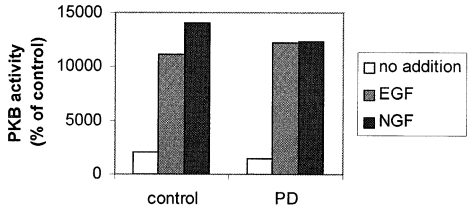

Fig. 2. Activation of PKB by EGF or NGF is not inhibited by PD98059. PC12 cells were serum-starved for 2 h and treated with EGF for 3 min or with NGF for 5 min (previously reported to be the peak times for PKB activity [16]) in the absence (control) or presence (PD) of PD98059. All three isoforms of PKB were immunoprecipitated and a kinase assay was performed as described in Section 2. The PKB activity in the control cells with no growth factor added was set at 100%. The graph shows an average of two independent experiments.

significantly affected GSK-3 activity relative to the control cells treated with PD98059. These data imply that ERK signalling is required for the inactivation of GSK-3 in response to EGF or NGF in PC12 cells.

We have previously shown a correlation between the activation of eIF2B and inactivation of GSK-3. Both the kinetics [7] and the pathways involved in the regulation were similar after EGF or NGF treatment of PC12 cells. A phospho-specific antibody for the GSK-3 phosphorylation site in eIF2Bε, Ser535 (Ser540 in the rabbit sequence), is now available. Therefore, we wished to examine the phosphorylation state

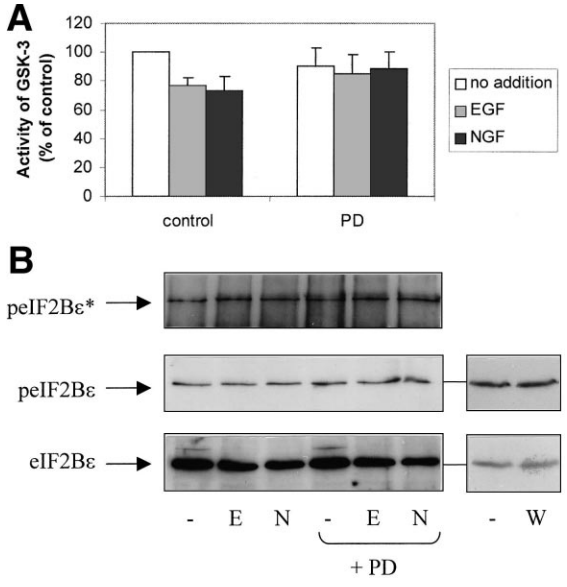

Fig. 3. Inactivation of GSK-3 by EGF or NGF is not affected by PD98059. A: The cells were serum-starved for 2 h and treated with EGF (60 min) or NGF (90 min) in the absence (control) or presence (control+PD) of PD98059. After immunoprecipitation of GSK-3, its activity was measured as described in Section 2. The graph shows an average of six independent experiments ($P < 0.005$). The error bars represent the standard error of the mean. B: Equal amounts of cell lysate were analysed by SDS–PAGE and Western blotting. Phosphorylation of Ser535, the GSK-3 site in eIF2Bε, was detected with a phospho-specific antibody, phosphorylation of the 'priming site' Ser539 was detected with a phospho-specific antibody and the total amount of eIF2Bε was detected with a polyclonal antibody raised against eIF2Bε ($n = 6$). eIF2Bε indicates the total amount of protein, peIF2Bε indicates eIF2Bε phosphorylated at Ser535, and peIF2Bε* indicates eIF2Bε phosphorylated at Ser539. −: no addition, E: EGF, N: NGF, W: wortmannin (100 nM, 2 h).

of this residue in PC12 cells after EGF or NGF treatment to show that GSK-3 does indeed play a role in the regulation of eIF2B in these cells. However, we were unable to detect a change in its phosphorylation at this site in response to EGF or NGF (Fig. 3B). The basal level of phosphorylation of Ser535 was also not affected by PD98059 or wortmannin. The only other identified phosphorylation site in eIF2Bε is Ser539. Using synthetic peptides, it was shown that this residue ('priming site') needs to be phosphorylated before GSK-3 is able to phosphorylate Ser535 [17,18]. To assess its possible role in regulating eIF2B activity, we examined whether phosphorylation of Ser539 was affected by EGF or NGF treatment. No change in the phosphorylation state of this site was detected under these conditions. These data suggest that EGF and NGF regulate eIF2B via a mechanism that does not involve changes in the phosphorylation of Ser535 or Ser539 in eIF2Bε.

4. Discussion

This report shows for the first time that activation of MEK is required for the stimulation of eIF2B specifically in response to EGF or NGF in PC12 cells. We have previously shown that the activation of eIF2B required a wortmannin-sensitive component. Our results show that PD98059 did not affect PKB activation and wortmannin hardly interfered with ERK signalling. We therefore conclude that in PC12 cells two distinct pathways are involved in the regulation of eIF2B activity.

Based on the present data and previous findings [7] it appears that both MEK- and PI 3-kinase-dependent pathways are also involved in the inactivation of GSK-3 in response to EGF or NGF. In CHO-T cells, the inactivation of GSK-3 is associated with the dephosphorylation of Ser540 (Ser535 in rat sequence), the GSK-3 phosphorylation site in eIF2Bε. Even though phosphorylation of Ser535 was detected in control PC12 cells, no dephosphorylation of Ser535 was observed in response to EGF or NGF, which indicates that in PC12 cells the PI 3-kinase- or MEK-dependent regulation of the activation of eIF2B by EGF or NGF is unlikely to involve GSK-3. The phosphorylation state of the 'priming site', Ser539, was also not affected by EGF or NGF treatment.

Since both the identified phosphorylation sites in eIF2Bε, Ser535 and Ser539, are not regulated by EGF or NGF in PC12 cells, it is not clear how the regulation of eIF2B activity is linked to the MEK/ERK pathway. It may involve the (de)-phosphorylation of another, as yet unidentified, site in eIF2Bε or in another subunit of the eIF2B heteropentameric complex.

Another important finding of this study is that in PC12 cells inactivation of GSK-3 in response to EGF or NGF occurs via the MEK and PI 3-kinase pathways. Involvement of the MEK pathway in the regulation of GSK-3 activity has been suggested before. Transfection of a dominant negative mutant of MEK in NIH/3T3 cells inhibited the EGF-induced decrease in GSK-3 activity [19]. This may be mediated by $p90^{rsk}$, since early in vitro studies showed that GSK-3 can be inactivated by $p90^{rsk}$, which is activated by ERK [20–22].

The absence of dephosphorylation of Ser535, although GSK-3 activity is decreased, is an interesting finding. This may reflect the relatively small change in GSK-3 activity (20–30%) in these cells: dephosphorylation of the GSK-3 site in eIF2Bε was only previously shown in insulin-treated

CHO-T cells, where the effect on GSK-3 is much larger (60–70% inactivation) [23,24]. However, it is also possible that additional regulatory inputs are important in the activation of eIF2B in PC12 cells under these conditions, e.g. the activation of a phosphatase. By analogy, in rat adipocytes, PDGF and insulin each inactivate GSK-3, but only insulin affects the downstream target glycogen synthase. The difference between the two stimuli is that insulin also activates protein phosphatase 1, which is apparently required for activation of glycogen synthase [25]. A similar situation may apply to the regulation of eIF2B by the different stimuli in PC12 cells, but it remains to be determined which phosphatase dephosphorylates Ser535 in eIF2Bε. Furthermore, a role for GSK-3 in the regulation of eIF2B has mostly been proposed for cells treated with insulin [3,6,26].

From this paper and previous work, it has become clear that the MEK/ERK pathway is important in the regulation of general protein synthesis and of several translation initiation factors. In PC12 cells, the EGF- or NGF-induced increase in protein synthesis is blocked by the MEK inhibitor PD98059, which coincides with inhibition of the phosphorylation of eIF4E, the formation of the eIF4F complex and the activation of eIF2B.

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